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ANTIBODY-DEPENDENT ENHANCEMENT OF DENGUE VIRUS GROWTH IN HUMAN MONOCYTES AS A RISK FACTOR FOR DENGUE HEMORRHAGIC FEVER

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Abstract. Serum specimens collected during a prospective study of dengue infections among schoolchildren in Bangkok were tested for their ability to enhance dengue 2 (DEN-2) virus growth in human monocytes in vitro. Two groups of dengue-immune sera were compared: 32 dengue antibody positive serum specimens from children who subsequently developed asymptomatic secondary dengue infections; and 9 dengue antibody positive serum specimens from children who subsequently developed severe symptomatic secondary dengue infections, 8 of which were clinically diagnosed as dengue hemorrhagic fever. Antibody-dependent enhancement of virus growth was quantitated by measurement of virus yields in supernatant fluids of normal human monocyte cultures that were infected with DEN-2 virus in the presence of undiluted test serum. Only 4 of 32 (12%) preinfection sera from asymptomatic children, but 6 of 9 (67%) preinfection sera from symptomatic children, had significant enhancing activity ($P < 0.001$). High serum DEN-2 antibody dependent enhancing activity is a significant (relative risk = 6.2) risk factor for severe illness among children in a dengue hemorrhagic fever endemic region. Dengue antibodies can be neutralizing and therefore protective, or they can be enhancing and increase the risk of dengue hemorrhagic fever.

Dengue hemorrhagic fever dengue shock syndrome (DHF DSS), the most severe manifestation of an acute dengue virus infection, is endemic among children in Southeast Asia. There are 4 serotypes of dengue viruses (DEN-1, -2, -3, and -4). Prior infection with a dengue virus of 1 serotype confers only transient immunity to infection with heterologous serotypes, but does give rise to antibodies broadly cross-reactive with virions of all 4 serotypes.[1,2] Pre-existing seroimmunity to dengue, as measured by conventional serologic assays, is an important risk factor for the development of severe manifestations of infection.[3,4] More than 95% of children <12 months of age hospitalized with severe dengue show evidence of a secondary (anamnestic) seroresponse. Dengue-naive children rarely develop severe manifestations of disease when acutely infected. Infants born to dengue-immune mothers are also at increased risk of severe disease until maternal antibodies wane.[5]

Antibody-dependent enhancement (ADE) of dengue virus growth in mononuclear phagocytes is thought to be the mechanism whereby pre-existing dengue antibodies confer excess risk.[6,7] Heterotype antibodies bind to, but do not neutralize, dengue virions. Heterotypic antibody-virion complexes attach to mononuclear phagocytes throuch Fc receptors on the cell surface.

In a 6 month prospective study of dengue infections among Bangkok schoolchildren, we demonstrated that existing seroimmunity to dengue, as measured by detection of serum hemagglutination-inhibiting antibodies, was a significant risk factor for development of DHF DSS among children who became acutely infected by a dengue virus.[8] In that study, 8 of 56 seropositive children who became infected by a dengue virus developed DHF/DSS; none of 46 previously seronegative children became seriously ill when infected. One additional seropositive child developed relatively severe illness but was not clinically diagnosed as having DHF/DSS.

In the current study, we sought to determine if differences in serum ADE activity in preinfection sera from seropositive children could be related to the severity of subsequent infection. We report here that, among dengue-immune chil-

dren, high serum DEN-2 ADE activity was a strong predictor of severe illness.

MATERIALS AND METHODS

Case studies

The study subjects were children in the Phibunprachasan School who participated in the 1980–1981 prospective study of dengue infections in Bangkok, Thailand.[8] Pre-infection serum specimens included in this study were from 9 children with secondary infection who developed clinical symptoms and 32 out of 39 children with secondary infections who were asymptomatic (there was an insufficient quantity of serum in the remaining 7 samples). For negative controls, 10 unselected preinfection sera from among 47 children with subsequent primary dengue infection were also examined (none had detectable dengue antibodies). All serum specimens were previously tested for their hemagglutination inhibition and neutralization activities against all 4 dengue serotypes as described by Burke and others.[8]

Preparation of virus stock

DEN-2 virus strain D80-616 was isolated from plasma from an infant DSS case in Bankok during the 1980 outbreak using the mosquito inoculation technique.[9] The virus was further propagated in LLC-MK$_2$ monkey kidney cell monolayers. Culture fluid containing virus was harvested, quantitated for the virus as plaque forming units (PFU),[10] and stored at −70°C until it was used in the infection-enhancement tests.

Macro-assay for DEN-2 antibody-mediated enhancement activity

Antibody-mediated enhancement activity of preinfection sera from appropriate study cases was measured using the method described by Brandt and others[11] with some modifications. Briefly, 5×10^5 cells of a 3-day-old culture of the human monocytic cell line U-937 were infected for 90 min at 37°C with DEN-2 virus strain D80-616 at the multiplicity of infection of 0.1 in the presence of heat-inactivated test serum at varying dilutions (1:10–1:10^6). The virus and serum mixture was removed and cells were washed 3 times before they were cultured in a 15 ml polystyrene centrifuge tube containing 2 ml RPMI 1640 with 10% heat-inactivated fetal calf serum, 200 mM glutamine, 200 μg/ml streptomycin, and 200 U/ml penicillin with an addition of test serum at various final dilutions. The cultures were kept in a CO_2 incubator at 37°C for 5 days with daily shaking for 2–3 sec. The cells and culture fluids were harvested after a centrifugation at 1,000 rpm for 10 min. Infection was quantitated by numeration of acetone-fixed cells reactive to DEN-2 polyclonal antibodies by an indirect immunofluorescent antibody test. Virus produced in culture fluids was also measured by quantitating dengue virus PFU in rhesus monkey kidney LLC-MK$_2$ cell monolayers. ADE is measured as an increase in the number of infected cells or a statistically significant increase of virus production in the fluids from cultures infected in the presence of the test human serum compared to those infected and cultured in the absence of test serum. Since the quantity of virus produced in cell cultures correlated with the number of U-937 cells infected (S. Kliks, personal communication), the enhancement in this assay was determined by comparing the number of infected cells in the presence of test serum to that of cells infected in the absence of human serum.

Micro-assay for DEN-2 antibody-mediated enhancement activity

This test was employed to measure ADE activity in undiluted human serum or in low serum dilutions as described by Eckels and others.[12] Human monocytes, freshly isolated by the elutriation method,[13] were used because of their ability to remain viable in the presence of undiluted or a low dilution of human serum. In this assay, infection was measured by counting the percentage of infected monocytes as well as quantitating infectious virus in culture fluids. The amount of virus produced in an infected monocyte culture correlated with the number of cells infected as detected by the immunofluorescent technique.

RESULTS

Determination of DEN-2 antibody-mediated enhancement activity in preinfection sera

We tested preinfection sera from children in the symptomatic and asymptomatic groups with

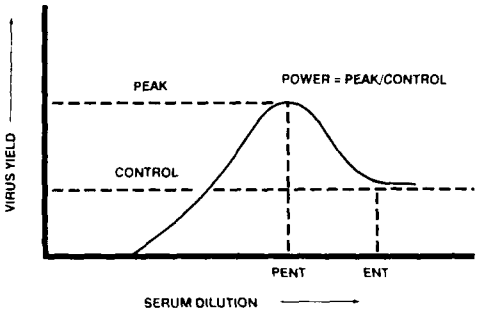

FIGURE 1. Schematic graph of DEN-2 virus yield vs. dilution of dengue immune serum in cultures of U-937 cells. CONTROL is the mean yield from cultures without added serum; PENT is the peak enhancement titer, or the titer at which virus yield is maximal for the serum tested; ENT is the enhancement titer or the highest dilution of the test serum which produces a yield significantly greater than the control yield; and POWER is the ratio of yield at peak enhancement titer divided by the control yield.

secondary dengue infections for their ability to enhance infection of DEN-2 virus strain D_{2}16 in U-937 cells. Preinfection sera from 10 children with primary dengue infection from the same study cohort were included in the test as negative controls. Serum dilutions < 1:10 were not tested due to their toxic effect on U-937 cells. Three enhancement parameters were measured: ADE titer (ENT), peak ADE titer (PENT), and power of enhancement (POWER). These parameters are diagrammed in Figure 1. In the experiment with U-937 cells, no DEN-2 infection was observed in cell cultures in the absence of test serum or in the presence of nonimmune sera from the control group; but DEN-2 infection was observed in all cultures with pre-secondary infection sera, indicating dengue infection enhancing activity (Table 1). Paradoxically, the mean DEN-2 ENT and PENT titers among the sera from the asymptomatic group were higher than those from the symptomatic group ($P < 0.05$; Table 2). In addition, the mean POWER in sera from the asymptomatic group was also higher than that from the symptomatic group ($P < 0.05$) when tested using diluted sera on U-937 cells (Table 2).

Analysis of the relationship between DEN-2 $PRNT_{90}$ and ADE activities

Although the DEN-2-enhancing activities (ENT, PENT, and POWER) among the children in the asymptomatic group were higher than those of the symptomatic group, we noted a basic difference in the enhancement profiles between the 2 groups. In the asymptomatic group, the peak enhancement was observed at the dilutions ≥ 1:40 among 75% (24 out of 32) of sera (Table 1). Those sera also exhibited strong neutralization in the preceding dilutions. In contrast, 77.8% (7 out of 9) of sera from the symptomatic group exhibited maximum enhancement at the lowest dilution tested (typically 1:10). It seemed appropriate therefore to retest all sera for ADE at the lowest possible dilution, most closely simulating the situation in vivo.

DEN-2 enhancement activity in undiluted sera

In order to estimate the effect of neutralizing and enhancing activities on DEN-2 infection in human monocytes in vivo, we tested the undiluted sera from the symptomatic and asymptomatic groups for their ability to either neutralize or enhance dengue infection in freshly isolated human monocytes with the microassay method. Ten preinfection sera from the primary infection group were included as normal serum controls. A slight infection, indicated by virus production of < 500 PFU per culture (0.16% of infected cells), was observed with control sera. Six out of nine sera from the symptomatic group (67%) exhibited infection greater than 2 standard deviations (SD) above the control mean value observed in normal sera (Fig. 2). One serum exhibited a degree of infection above the mean of normal serum controls, but ≤ 2 SD above the mean. The remaining 2 sera with high DEN-2 $PRNT_{90}$ (as measured in LLC-MK_2 cells) also exhibited neutralization in human monocytes (Fig. 2).

Of the 32 sera in the asymptomatic group, only 4 (9.3%) exhibited ADE resulting in infection > 2 SD above the control mean. Three were associated with an infection level comparable to that observed with normal sera. However, 25 out of 32 sera from the asymptomatic children (78%) exhibited infection levels < 2 SD below the mean value of normal controls.

Analysis of risk for DHF/DSS from DEN-2 enhancement activity

Children whose sera neutralized DEN-2 virus in human monocyte cultures, regardless of their DEN-2 enhancing activities measured at higher

TABLE 1

Disease severity among 3 study groups and the neutralizing and enhancing activities in their preinfection sera

Case	Symptoms*	DEN-1 PRNT†	DEN-2 PRNT	DEN-3 PRNT	DEN-4 PRNT	DEN-2 ENT‡	DEN-2 PENT§	DEN-2 POWER¶	DEN-2 INF**
I. Asymptomatic primary (n = 10)									
3218	0	0	0	0	0	0	0	0	2.30
2605	0	0	0	0	0	0	0	0	1.80
2698	0	0	0	0	0	0	0	0	2.07
2737	0	0	0	0	0	0	0	0	2.04
2514	0	0	0	0	0	0	0	0	2.70
2544	0	0	0	0	0	0	0	0	1.82
887	0	0	0	0	0	0	0	0	1.90
249	0	0	0	0	0	0	0	0	2.60
410	0	0	0	0	0	0	0	0	1.45
1866	0	0	0	0	0	0	0	0	2.30
II. Asymptomatic secondary (n = 32)									
2635	0	5	5	375	5	160	10	4.0	1.75
3163	0	5	15	5,000	5	160	40	4.3	0.70
2604	0	5	13	750	178	40,960	40	14.5	1.58
573	0	700	12	5	5	160	40	2.5	2.50
2202	0	150	35	680	41	1,560	40	21.5	0
2263	0	130	5	1,300	110	40,960	160	34.3	0
2272	0	800	80	200	10	640	40	11.9	1.76
2781	0	2,000	15	5	5	160	40	3.1	3.09
2814	0	5	750	12	5	640	40	18.6	0
5	0	5	300	5	5	640	40	5.3	1.35
147	0	5	5	5	215	160	10	4.5	2.79
215	0	21	550	5	5	10,240	40	16.5	0
2881	0	5	5	5	160	40	10	1.1	3.20
57	0	600	400	200	56	10,240	640	13.4	0
797	0	375	40	5	10	10,240	40	9.5	0.95
124	0	770	60	13	12	10,240	160	6.9	0
1203	0	70	83	525	37	10	10	6.0	1.21
1485	0	5	570	5	5	10,240	160	22.1	0
181	0	17	22	130	1,500	2,560	40	18.5	0
2537	0	225	55	1,450	20	2,560	160	25.3	0
418	0	15	13	2,100	5	2,560	10	6.2	0.94
846	0	14	30	5	68	2,560	40	19.2	1.20
2501	0	5	5	16	43	160	40	9.8	3.24
2340	0	15	10	12	640	2,560	40	5.9	1.60
1397	0	32	135	350	5	10,240	40	13.2	0.60
137	0	90	85	5	5	10,240	10	7.4	0
1371	0	47	600	340	74	160	40	5.9	0
880	0	60	1,280	18	210	10,240	160	31.4	0
1220	0	5	5	5	72	160	40	3.5	2.08
626	0	11	26	550	58	640	10	9.6	0
1511	0	5	5	5	44	160	10	6.0	2.40
125	0	10	50	640	5	2,560	40	7.3	0
III. Symptomatic secondary (n = 9)									
3295	3	20	10	110	50	160	40††	4.7	3.62
1935	3	700	50	110	5	640	10	4.0	2.67
2494	3	1,620	20	29	5	160	40	8.7	2.75
2354	3	520	600	88	10	160	10	1.9	0
818	2	5	640	5	11	10,240	40	18.5	0
892	3	1,130	5	11	21	40	10	0.5	2.40
29	1	5	5	5	20	160	10	1.0	5.10
333	3	150	11	11	5	10	10	2.6	5.40
408	3	1,380	45	70	5	10	10	1.3	4.80

* Dengue symptoms: 0 = no symptoms, 1 = fever, 2 = DHF, not hospitalized, and 3 = DHF, hospitalized.

† PRNT = 50% plaque reduction neutralization titer as measured on LLC-MK$_2$ cells.

‡ ENT = Enhancement titer on U-937 cells.

§ PENT = *Peak enhancement titer on* U-937 cells.

¶ POWER = Power or fold of enhancement on U-937 cells compared to yield from cultures without added human sera.

** INF = Infection yield in freshly elutriated human monocytes cultured in the presence of undiluted serum. $\log_{10}$ of plaque forming units per culture.

†† Lowest dilution tested.

TABLE 2

Comparison of dengue $PRNT_{50}$ titers and DEN-2 enhancing activities between symptomatic and asymptomatic children with secondary infections

	DEN-1 PRNT*	DEN-2 PRNT*	DEN-3 PRNT*	DEN-4 PRNT*	DEN-2 ENT*	DEN-2 PENT*	DEN-2 POWER
Symptomatics (n = 9)	2.2 ± 1.0	1.5 ± 0.8	1.4 ± 0.5	1.0 ± 0.4	2.1 ± 0.9	1.2 ± 0.3	4.8 ± 5.7
Asymptomatics (n = 32)	1.5 ± 0.8	1.6 ± 0.7	1.7 ± 1.4	1.4 ± 0.7	3.1 ± 0.9	1.6 ± 0.4	11.3 ± 8.5
P value	0.09	0.78	0.24	0.03	0.02	0.01	0.01

* $\log_{10}$ value.
PRNT = 50% plaque reduction neutralization titer as measured on LLC-MK_2 cells.
ENT = Enhancement titer measured on U-937 cells.
PENT = Peak enhancement titer measured on U-937 cells.
POWER = Power or fold of enhancement measured on U-937 cells.

dilutions, had a relatively low risk (0.15) for DHF DSS (Table 3). The Mantel-Haenszel chi-square statistic was 10.9 ($P < 0.001$), indicating a significant association between DEN-2 neutralization and lower incidence of DHF DSS.

In contrast, those children whose undiluted sera enhanced DEN-2 infection in human monocytes, regardless of their original DEN-2 $PRNT_{50}$, had a relative risk of 6.2 (Table 3). The Mantel-Haenszel chi-square statistic was 9.53 ($P < 0.005$), indicating a significant association between DEN-2 enhancement and DHF DSS. The attributable risk of DEN-2 enhancement toward the development of DHF DSS was 0.84.

Interrelationships among neutralization, infection enhancement, and severity of symptoms

Since DEN-2 infection enhancement by undiluted serum was associated with the risk of developing symptoms during subsequent infection, we further analyzed the relationship between the degree of enhancement and the severity of dengue illnesses. We also determined the relationship between the risk index (infection enhancement by undiluted serum) and other conventional serologic properties.

We labeled the 4 observed outcomes of the

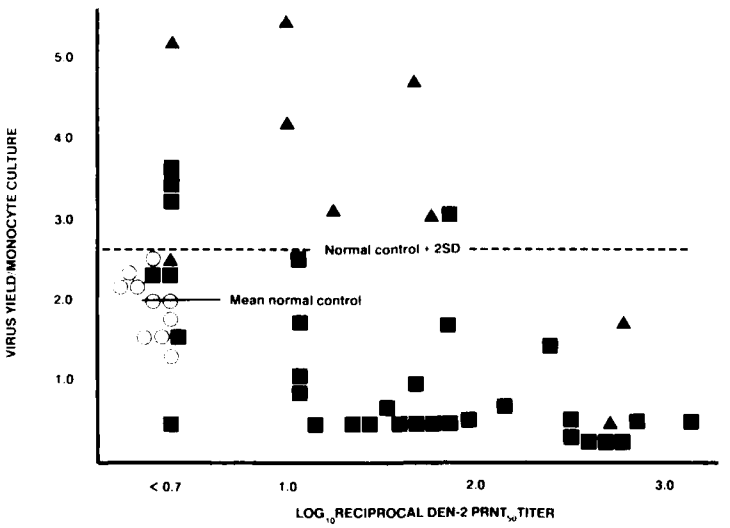

FIGURE 2. Graph of relationship between DEN-2 $PRNT_{50}$'s (as measured on LLC-MK_2 cells) vs. DEN-2 virus yields in cultures of fresh elutriated human monocytes to which undiluted sera were added. Control sera, from patients who subsequently experienced asymptomatic primary infections, are shown as open circles (○); sera from patients who subsequently experienced asymptomatic secondary infections are shown as closed squares (■); and sera from patients who subsequently experienced symptomatic secondary infections are shown as closed triangles (▲).

severity of dengue illnesses—asymptomatic, dengue fever, DHF but not hospitalized, and hospitalized for DHF—as 0, 1, 2, and 3, respectively (Table 1). We then analyzed the relationship between this variable and the degree of infection in human monocytes cultured in tested undiluted serum, and all other enhancement parameters and $PRNT_{90}$ against DEN-1, -2, -3, and -4 (Table 1) using the Spearman rank correlation method. Severity of symptoms correlated with yield of DEN-2 virus in monocyte cultures supplemented with undiluted preinfection sera ($r = 0.420$, $P < 0.01$). Enhancement outcome (>2 SD) was also shown by the Spearman rank correlation test to be correlated with development of dengue symptoms ($r = 0.526$, $P < 0.001$). Preexisting neutralization of DEN-1 showed a marginal correlation ($r = 0.332$, $P < 0.05$) with dengue symptoms.

Neutralization of infection in human monocytes by undiluted serum was negatively correlated with symptoms by rank correlation analysis ($r = 0.372$, $P < 0.01$). This variable correlated with DEN-2 $PRNT_{90}$ as measured in LLC-MK$_2$ cells ($r = 0.687$, $P < 0.001$), which in turn covaried with DEN-2 enhancement titer (ENT) ($r = 0.424$, $P < 0.01$), peak enhancement titer (PENT) ($r = 0.366$, $P < 0.05$).

TABLE 3

Risk analysis of severe illness for patients with sera showing DEN-2 neutralizing or enhancing activities as measured with undiluted sera on fresh human monocytes

DEN-2 neutralization	Symptomatic	Asymptomatic
Yes	2	25
No	7	7
DEN-2 Enhancement	**Symptomatic**	**Asymptomatic**
Yes	6	4
No	3	28

Relative risk of neutralization = 0.15
Relative risk of enhancement = 6.20
Attributable risk = 0.84

DISCUSSION

The results of this study provide direct evidence that antibody-dependent enhancement of dengue virus growth in mononuclear phagocytes is central to the pathogenesis of dengue hemorrhagic fever. The presence of DEN-2 infection enhancing antibodies in undiluted serum is a specific and powerful risk factor for the development of DHF DSS during a subsequent secondary dengue infection, while the presence of DEN-2 neutralizing antibodies in serum predicts a subsequent asymptomatic secondary infection.

Serum dengue antibody dependent enhancing activity can be described by 3 measurements: the maximal dilution at which enhancement can be detected, or the "enhancement titer"; the dilution at which enhancement is most readily apparent, or the "peak enhancement titer"; and the relative yield compared to control sera, in terms of the number of infected cells or the amount of virus in culture supernatant fluids, at the peak enhancement titer (the relative yield is quantitated as the "power of enhancement"). These measurements are diagrammed in Figure 1. In our initial experiments using U-937 cells, we measured enhancement titers, peak enhancement titers, and powers of enhancement. Oddly, DEN-2 enhancement titers, peak enhancement titers, and the power of enhancement were greater using preinfection sera from asymptomatic cases than from symptomatic cases, apparently refuting the hypothesis that enhancement is related to the severity of disease. However, the basic shapes of the enhancement activity curves between the 2 groups differed. Enhancing activity at the lowest dilution tested in the U-937 assay system (1:10) was greater using sera from symptomatic cases than when using sera from asymptomatic cases.

We therefore devised an alternate test system to extend the curves to measure enhancing activity of undiluted sera. Cultures of freshly elutriated normal human monocytes were found to work well. In this system, virus yields (power of enhancement) using undiluted sera from symptomatic cases were substantially greater than those using sera from asymptomatic cases. In retrospect, it is apparent that use of undiluted sera on normal human monocytes is the appropriate system for measuring physiologically relevant ADE: blood mononuclear cells are bathed in undiluted serum in vivo.

Immune serum can have 2 competing effects on dengue virus growth in cultures of Fc-receptor-bearing cells: neutralization or enhancement (Fig. 3). If neutralizing activity is high, no enhancement is observed. Conversely, high enhancing activity may mask the presence of low-level neutralization. All 7 sera from symptomatic cases in this series which showed enhancement in monocyte cultures had low-level neutralizing

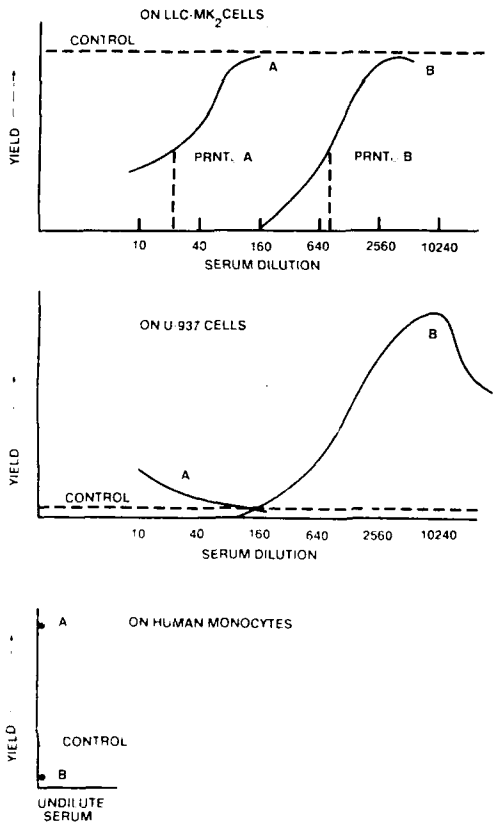

FIGURE 3. Schematic graph of DEN-2 virus yields vs. serum dilutions when tested on LLC-MK, monkey kidney cells (top), on U-937 cells (center), or on human monocytes (bottom). A shows the pattern typical of sera from patients who subsequently experienced symptomatic infections. B shows the pattern typical of sera from patients who subsequently experienced asymptomatic infections. PRNT, 50% plaque reduction neutralization titer.

titers (1:10–1:50) when tested in LLC-MK, cells, an epithelioid cell line derived from monkey kidneys. Because LLC-MK, cells lack Fc receptors, assays on these cells selectively measure neutralizing activity and not enhancing activity.

One of the limitations of this report is that viruses were not isolated from the schoolchildren who became infected during the study period. DEN-2 virus is regularly associated with DHF DSS in Bangkok. During the epidemic season when sera were collected (1980), 73% of all isolates from DHF DSS cases at Bangkok Children's Hospital were serotype 2, 21% were serotype 1, and serotypes 3 and 4 accounted for the remainder.[14] The strain of virus used in the enhancement assays (D_{80}616) was isolated from the blood of a patient with DHF in Bangkok in 1980; it was therefore probably serotype- and strain-homotypic with the viruses that infected most of the study cases. We cannot exclude the possibility that some of the study children were infected by viruses other than serotype 2. Indeed, preinfection sera from 2 of 9 symptomatic children had high DEN-2 neutralizing activity; these children may have been acutely infected by DEN-1 instead of DEN-2 during the study period. If these cases had been excluded from the analysis, the association of serum DEN-2 enhancing activity and subsequent illness would have been essentially perfect. Serum from 1 additional symptomatic case lacked enhancing activity as defined by a yield >2 SD above the mean yield from cultures with nonimmune sera. Nonetheless, this sera did produce a 3-fold greater yield than the mean of cultures with nonimmune sera.

If the results for DEN-2 ADE activity in sera from children from Bangkok in 1980–1981 hold for other regions, then it may be possible to identify prospective subjects who are at high risk for DHF DSS. Localized populations in tropical regions which are at high risk for DHF DSS can be identified by measuring the prevalence of DEN-2 ADE activity in undiluted serum. Populations found to be at risk of epidemic DHF DSS could then be protected by intensified mosquito control efforts. Similarly, sera from recipients of candidate dengue vaccines should be tested for serum DEN-2 ADE activity to determine if the vaccines have been rendered dengue immune or placed at high risk of DHF DSS.

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